interest for its effects on the physical properties of biological membranes. In model membrane studies ceramide has been shown to promote phase separation and domain formation, non-lamellar phases, and enhanced membrane permeability. We recently reported the synthesis and photochemical characterization of long-chain ceramides caged with a 6-bromo-7-hydroxycoumarin group. These derivatives were readily dispersed in aqueous solution, successfully delivered into cells and uncaged therein. Caged compounds such as these can be released in their biologically active form with a high degree of spatial and temporal control. These characteristics are attractive in applications where well-defined amounts of ceramide must be generated. Previous work has largely relied on exogenous delivery of synthetic short-chain ceramides or sphingomyelinase treatments where enzyme activity can be difficult to control. Further studies are currently under way in HL-60 cells using the caged compounds to probe the role of long-chain ceramides in pro-apoptotic pathways. Concurrently, we have incorporated caged ceramides into supported lipid bilayers as part of ongoing work to model ceramide's mechanical and morphological effects in membranes.

Ryanodine Receptors

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The Ryanodine Receptor N-Terminal Disease Hot Spot Intersubunit Interface is Disrupted by Channel Opening and Affected by Disease Mutations Acting via Long-Range Structural Changes

Lynn Kimlicka, Kelvin Lau, Ching-Chieh Tung, Filip Van Petegem.
University of British Columbia, Vancouver, BC, Canada.

Ryanodine Receptors (RyRs) are intracellular calcium-release channels acting as one of the key regulatory elements in the excitation-contraction coupling. More than 350 mutations have been found in RyRs that are known to underlie severe genetic diseases. Mutations in the skeletal muscle isoform (RyR1) are associated with malignant hyperthermia (MH) and central core disease (CCD), while mutations in the cardiac isoform (RyR2) cause catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia (ARVD). Most mutations confer a gain of function, but the precise mechanisms that explain enhanced channel opening up to the molecular scale have remained elusive. Here we present pseudo-atomic models of the N-terminal disease hot spot in the open and closed states of the RyR, along with crystal structures of several disease mutants. The data show that the intersubunit interfaces formed by tetrameric N-terminal disease hot spots are disrupted upon channel opening in wild-type RyRs. This intersubunit interface harbors 19 disease mutations, the largest cluster within the N-terminal region, indicating the vulnerability of this interface in channel regulation. We present crystal structures and thermal stabilities of nine disease mutants located at other interfaces. The effect of most mutations are destabilizing to the protein, with decreases in melting temperatures as large as ~10°C. Buried disease mutations cause structural changes to the intersubunit interface, while mutations affecting ionic pairing at the intra-subunit interface significantly alter relative domain orientations. Mutations far away from the intersubunit interface can thus affect these contacts via long-range conformational changes. These results illuminate the intersubunit interface between N-terminal disease hot spots as a prime target for disease mutations through direct or indirect conformational changes.

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Conformational Dynamics Inside Amino-Terminal Disease Hotspot of Ryanodine Receptor

Xiaowei Zhong[1], Ying Liu[2], Xing Meng[2], Ruiwu Wang[1], Filip Van Petegem[3], Terence Wagenknecht[2], S.R. Wayne Chen[1], **Zheng Liu**[2].
[1]Departments of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada, [2]Wadsworth Center, New York State Department of Health, Albany, NY, USA, [3]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada.

The amino-terminal region of both the skeletal and cardiac ryanodine receptor is a disease mutation hotspot. Previously, a crystal structure of a RyR1 fragment (amino acids 1-559) was solved and folded into three separate structural domains, A, B and C. Docking of the modeled fragment into a cryo-EM map of the full-length RyR1 has placed the ABC domains in a central vestibule in the cytoplasmic assembly. In the present study, we constructed a full-length RyR2-GFP chimera, with GFP inserted after residue Glu-310. The cryo-EM structure of $RyR2_{E310\text{-}GFP}$ clearly showed an extra mass in the domain B, which directly confirms the docking model. To probe the conformational changes in the N-terminal region of the channel, we generated three FRET pairs by inserting CFP or YFP after residues His-29, Glu-310, or Lys-441. Conformational dynamics inside the N-terminal region were analyzed using three dual-insertion pairs ($RyR2_{H29\text{-}CFP/E310\text{-}YFP}$, $RyR2_{H29\text{-}CFP/Y441\text{-}YFP}$, and $RyR2_{E310\text{-}YFP/K441\text{-}CFP}$) as well as in three co-expression single-insertion pairs ($RyR2_{H29\text{-}CFP}$/$RyR2_{E310\text{-}YFP}$, $RyR2_{H29\text{-}CFP}$/$RyR2_{Y441\text{-}YFP}$, and $RyR2_{E310\text{-}YFP}$/$RyR2_{K441\text{-}CFP}$). Our results indicated that upon RyR channel activation by caffeine, domains B and C move apart, whereas distances between domains A and B, and between A and C are almost unchanged inside one subunit of RyR. One other note of interest is that domain A moves away from both domains B and C in the neighboring subunits; meanwhile the distance between domains B and C from two neighboring subunits remains unchanged.

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Role of Amino-Terminal Half of the S4-S5 Linker in the RyR1 Channel Gating

Takashi Murayama[1], Nagomi Kurebayashi[1], Toshiharu Oba[2], Hideto Oyamada[3], Katsuji Oguchi[3], Takashi Sakurai[1], Yasuo Ogawa[1].
[1]Juntendo University School of Medicine, Tokyo, Japan, [2]Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, [3]Showa University School of Medicine, Tokyo, Japan.

The type 1 ryanodine receptor (RyR1) is a Ca^{2+} release channel found in the sarcoplasmic reticulum of skeletal muscle and plays a pivotal role in excitation-contraction coupling. The RyR1 channel is activated by a conformational change of the dihydropyridine receptor upon depolarization of the transverse tubule, or by Ca^{2+} itself, i.e., Ca^{2+}-induced Ca^{2+} release (CICR). The molecular events transmitting such signals to the ion gate of the channel are unknown. The S4-S5 linker, a cytosolic loop connecting the S4 and S5 transmembrane segments in six-transmembrane type channels, forms α-helical structure and mediates signal transmission in a wide variety of channels. To address the role of S4-S5 linker in the RyR1 channel gating, we performed alanine substitution scan of amino (N)-terminal half of the putative S4-S5 linker (Thr^{4825}-Ser^{4829}) that exhibits high helix probability. The mutant RyR1 was expressed in HEK cells and the CICR activity was investigated by caffeine-induced Ca^{2+} release, single-channel current recordings, and [^{3}H]ryanodine binding. Four mutants (T4825A, I4826A, S4828A and S4829A) had reduced CICR activity without changing the Ca^{2+} sensitivity, whereas the L4827A mutant formed a constitutive active channel. T4825I, a disease-associated mutation for malignant hyperthermia, exhibited enhanced CICR activity. An α-helical wheel representation of the N-terminal S4-S5 linker provides a rational explanation to the observed activities of the mutants. These results suggest that N-terminal half of the S4-S5 linker may form α-helical structure and play an important role in the RyR1 channel gating.

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Oligomerisation of the Human Cardiac Ryanodine Receptor Amino-Terminus

Monika K. Seidel, F.A. Lai, Spyros Zissimopoulos.
Cardiff University, Cardiff, United Kingdom.

The cardiac ryanodine receptor (RyR2) mediates the release of calcium from the sarcoplasmic reticulum of cardiac myocytes. The functional channel is composed of four identical subunits with the C-terminal part comprising the transmembrane domain predicted to form the Ca^{2+}-conducting pore. The large N-terminal cytoplasmic portion of RyR2 is believed to serve as a scaffold for interaction with accessory proteins, ions and other regulatory molecules. RyR2 channel gating is regulated by a complex network of inter- and intra-subunit interactions between discrete structural domains. It has been proposed that disruption of inter-domain cross-talk results in abnormal RyR2 channel function, as observed in catecholaminergic polymorphic ventricular tachycardia (CPVT) and heart failure.

Here, we report that the RyR2 amino-terminus, containing one of the three CPVT-associated mutation hot spots, is capable of self-association. Chemical cross-linking of an RyR2 N-terminal fragment (BT4L; residues 1-906) indicated that it can assemble into tetramers. Moreover, BT4L expressed in mammalian HEK293 cells was found to form tetramers through endogenous disulphide bonds. We undertook a site-directed mutagenesis approach to identify the cysteines involved in BT4L disulphide bond formation, whereby cysteine residues were substituted by serine. The BT4L Cys361 Ser mutant did not form DTT-sensitive tetramers, suggesting that Cys361 participates in disulphide bond formation. When BT4L was co-expressed with full-length RyR2 in HEK293 cells it translocated from the cytosol to the

microsomal fraction. The functional significance of RyR2 N-terminus self-association was studied by [^{3}H]ryanodine binding assays. We found that the BT4L fragment activates the channel at low Ca^{2+} concentrations most likely by disrupting inter-subunit N-terminal self-association within the tetrameric channel. Our findings suggest that the RyR2 N-terminus regulates channel function through inter-subunit interactions. This work was supported by the British Heart Foundation.

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STED Nanoscopy of Cardiac RyR2 Clusters and Sub-Structure Analysis After Myocardial Infarction

Tobias Kohl[1], Ulrich Parlitz[2], Marcel Lauterbach[3], Hoang-Trong Minh Tuan[4], George S.B. Williams[5], Volker Westphal[3], M. Saleet Jafri[5], W.J. Lederer[5], Stefan Luther[2], Stefan W. Hell[3], **Stephan E. Lehnart**[1].
[1]Dept. of Cardiology and Pulmonology, University Medical Center, Göttingen, Germany, [2]Biomedical Physics Group, Max Planck Institute for Dynamics and Self-Organization, Göttingen, Germany, [3]Dept. of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, [4]School of Systems Biology, George Mason University, Manassas, VA, USA, [5]Center for Biomedical Engineering and Technology, University of Maryland, Baltimore, MD 20742, Baltimore, MD, USA.

Cardiac ryanodine receptor (RyR2) Ca^{2+} release channels occur as multi-channel clusters at subcellular Ca^{2+} release sites in cardiomyocytes. RyR2 clusters are thought to underlie important signal amplification mechanisms including coupled gating of physically associated Ca^{2+} release channels. The nature of RyR2 channel organization in intact intracellular channel clusters is not known.

We have investigated the nanostructure of RyR2 clusters using Stimulated Emission Depletion (STED) nanoscopy with ~60 nm lateral resolution and indirect immunofluorescence in cardiomyocytes. We compared RyR2 clusters in adult cardiomyocytes from sham hearts with hearts 8 weeks after myocardial infarction (post-MI).

Peripheral RyR2 clusters were detected at a density of 1.3 $\pm$ 0.2 clusters per μm^2 in control cells (2237 clusters) and 1.6 $\pm$ 0.1 in diseased cells (1752 clusters). Individual clusters were analyzed by incrementally detecting signal edges which defined intra-cluster substructures and intensity peaks. Within individual clusters, nearest-neighbour distances between intensity peaks showed an asymmetric distribution with a maximum at 100 $\pm$ 1 nm in healthy cardiomyocytes. In post-MI cells this distribution showed a left shift with a maximum at 85 $\pm$ 3 nm ($p < 0.05$ Mann-Whitney test; mean distance sham 150.6 $\pm$3.2 nm vs post-MI 129.5 $\pm$ 2.7 nm).

In conclusion, immuno-labeled cardiac RyR2 clusters showed distinct non-randomly organized sub-structures as evidenced by characteristic intra-cluster peak-to-peak distances. Our data suggest that continuous RyR2 clusters contain substructures which occur repeatedly as densely organized groups of RyR2 channels. In addition, we showed altered RyR2 clusters after myocardial infarction with smaller intra-cluster peak-to-peak distances which implies tighter channel packing. We are preparing a working mathematical model of normal RyR2 cluster function and the role of potential substructures and their changes in heart disease.

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Mapping of the ATP Binding Sites in RyR1 Channel by Photoaffinity Labeling

Olga B. Popova[1], Tina P. Tran[1], Mariah R. Baker[2], Thao P. Tran[1], Irina I. Serysheva[1].
[1]The University of Texas Health Science Center at Houston Medical School, Houston, TX, USA, [2]Baylor College of Medicine, Houston, TX, USA.

Adenine nucleotides are basic modulators of Ca^{2+} release channels from sarcoplasmic reticulum, ryanodine receptors (RyRs). Similar to several other ion channels, ATP acts as a non-hydrolyzed ligand for activation of RyR1 with EC_{50} in the millimolar range: it enhances Ca^{2+} release from SR, increases ryanodine binding, and stimulates Ca^{2+}release through RyR1 channels incorporated into bilayers. The activation effect of ATP is likely to occur via its binding to one or more molecular sites on RyR1. However, the number and the localization of these sites have not yet been determined. We used $2N_3$ATP-2', 3'-Biotin-LC-Hydrazone (BioATP-HDZ), a photo-reactive ATP analog, as a probe for identification of the ATP-binding sites and for their location within the polypeptide chain of the RyR1 protein. We found that BioATP-HDZ binds specifically to the purified RyR1 upon photo-activation by ultraviolet light. The covalent binding of BioATP-HDZ was inhibited by unlabeled ATP with IC_{50}= 0.6 $\pm$ 0.2 mM. This is in agreement with the reported EC_{50} value for activation of RyR1 with ATP (~0.5 mM) and suggests a similar affinity for BioATP-HDZ. The BioATP-HDZ-labeled SR membranes and purified RyR1 were subjected to limited proteolysis by trypsin, followed by SDS-PAGE analysis, and the labeled proteolytic fragments were identified with IR800Dye-streptavidin using an Odyssey near-infrared scanner. *N*-terminal sequence analysis of the labeled proteolytic fragments revealed three putative sequences of RyR1 protein that possibly constitute ATP-binding site(s) in the channel: 95 kDa (amino acids 426-1508), 44 kDa (amino acids 2402-2795) and 70 kDa (amino acids 4476-5037). Only one fragment includes the consensus motifs (amino acids 427-432, 699-704 and 1195-1200) for potential nucleotide-binding sites found in the primary sequence of RyR1.

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Estimation of Pore Geometry of RyR1 using Lanthanide Ruler

Laszlo Csernoch[1], Janos Almassy[1], Sandor Sarkozi[1,2], Balazs Lukacs[1], Istvan Jona[1,2].
[1]Department of Physiology, University of Debrecen, Debrecen, Hungary, [2]Research Center for Molecular Medicine, University of Debrecen, Debrecen, Hungary.

It was shown previously that the Ca analogue Gd inhibits RyR1 gating symmetrically with a Kd about 5.5 microM and Hill coefficient (nH) of 4 both on *cis* and *trans* side using single channel electrophysiology. We further tested the RyR1-lanthanide interaction using two lanthanides - having an ionic radii between Ca^{2+} and Gd^{3+} - by bilayer measurements and ryanodine binding experiments. *Cis* inhibition of RyR1 by Eu was characterized by a binding constant of Kd=167 $\pm$ 5 nM and an nH of 2 $\pm$ 0.1 while *trans* inhibition exhibits Kd=4.8 $\pm$ 0.2 microM and nN of 5.2 $\pm$ 1.2. The inhibition constants for Sm on the *cis* side are Kd=64.3 $\pm$ 2.5 nM and nH=2.2 $\pm$ 0.2 while on the *trans* side Kd=6.15 $\pm$ 0.13 microM and nH=4.68 $\pm$ 0.45. Inhibition by Eu and Sm are potential and polarity dependent in contrast to Gd due to the differences in ionic radii of these lanthanides. Increasing the ionic radius from 0.938 (Gd) to 0.964 (Sm) increased the binding affinity from 5.6 microM to 64.3 nM revealing that the size of Ca binding pocket is only slightly higher than the ionic radius of Sm. Ryanodine (Ry) binding experiments revealed that lanthanides bind - at least partially - to the regulatory Ca binding site because the dose response curve of 3H Ry binding starts with an increase of Ry binding, which amounts to about 40% for Eu and 70% for Sm of basic Ry binding. A model has been proposed for one possible spatial arrangement of lanthanide and calcium binding sites of the RyR1 pore based on the ionic radii of Ca and the tested lanthanides. Supported by OTKA 81923.

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S-Adenosyl-L-Methionine Induced RyR2 Subconductance: Evidence for an Allosteric Mechanism

Edward M. Balog, Angela J. Gaboardi.
Georgia Insitute of Technology, Atlanta, GA, USA.

We previously reported (Kampfer & Balog *Biochem* 49:7600, 2010) that S-adenosyl-l-methionine (SAM) can act as a RyR2 channel regulatory ligand in a manner independent from its recognized role as a biological methyl group donor. Channel activation appeared to arise from the interaction of SAM with a RyR2 adenine nucleotide binding site. In addition to its ability to activate RyR2, single channel recordings revealed distinct effects of SAM on RyR2 conductance, which we explored here in greater detail. The effects of SAM on native RyR2 channel conductance in symmetric cesium methanesulfonate were dependent on SAM concentration and holding potential. At negative potentials, *cis* SAM induced a single, clearly resolved subconductance state (~2/3 full conductance). The proportion of SAM induced subconductance openings, as a proportion of all openings (P_{sub}/P_o), increased with decreasing negative potential. Kinetic analysis revealed that changes in the SAM off rate accounted for the voltage dependence of the transitions between the full open and SAM induced subconductance state. In contrast, at positive potentials SAM caused a striking reduction in channel openings with no distinct effect on channel conductance. Inconsistent with a simple pore block mechanism was the finding that the prevalence of the subconductance state was unaffected by varying the cesium concentration gradient across the bilayer. Furthermore, ATP but not 4-chloro-m-cresol, interfered with the effects of SAM at both negative and positive potentials, suggesting ATP competition with SAM for